Functions of fibroblast growth factors and their receptors

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Fibroblast growth factors were first characterized twenty years ago as mitogens of cultured fibroblasts. Despite a wealth of data from experiments *in vitro*, insights have begun to emerge only recently on the normal function of these growth factors in mice and humans, as a result of studies of natural and experimental mutations in the factors and their receptors.

The FGF family of ligands

The fibroblast growth factor (FGF) family has grown since the first characterization of FGF as a fibroblast mitogen [1], to a current membership of nine, FGFs 1–9. High resolution X-ray structures of crystals of both FGF-1 and FGF-2 have been reported; these reveal a 'β trefoil' topology, comprising 12 strands linked to form a three-fold symmetrical structure [2]. On the basis of sequence conservation, it seems very likely that all members of the FGF family have related three-dimensional structures. At least some members of the family are also highly conserved in vertebrate evolution: amphibian homologues of FGF-2, FGF-3 and FGF-4 have been described [3–5]. There are now good descriptive data available on the expression of most FGF family members in the mouse (for example, see [6,7]), and less extensive, although generally corroborative, data for other species. Taken together, this evidence reveals two features of the developmental gene expression of the FGF family: firstly, each member exhibits a characteristic pattern of expression, and secondly, viewed as a whole, the FGFs are expressed at many stages and places in the both the developing and the mature organism.

The biological effects of the FGFs are mediated by association with three biochemically distinct partners: heparan sulphate oligosaccharides [8,9], a low-affinity transmembrane FGF-binding protein [10], and high-affinity transmembrane FGF receptors of the tyrosine kinase class. Transfection and reconstitution experiments (for example [11,12]) have shown that intracellular signal transduction is triggered by activation of FGF receptor tyrosine kinase activity. Activation is brought about by receptor oligomerization, which is mediated by the association of heparin with the ligand (FGF) [13] and of the ligand with the receptor; it is also possible that heparin associates with the receptor itself [14]. The role of the low-affinity transmembrane FGF-binding protein is at present obscure, although sequence considerations [10] suggest that it is more likely to play a role in the cellular uptake of FGF than to participate directly in pathways that transduce the FGF signal.

The transmembrane tyrosine kinase class of FGF receptors, as the main agents of signal transduction, are clearly essential for the biological function of FGFs. Four unlinked genes that encode FGF receptors, *FGFR1–FGFR4*, are currently known; the amino-acid sequences encoded by these four genes have related features (reviewed in [15]). The cytoplasmic region is principally composed of the tyrosine kinase domain, and it contains a number of consensus tyrosine residues that could act as phosphate acceptors for autophosphorylation. Most, but not all, of these residues are conserved amongst the FGF receptor family.

The receptor extracellular region comprises three domains, IgI–IgIII, each of which exhibits clear hallmarks of the immunoglobulin superfamily of structural modules (reviewed in [16]). IgI and IgII are separated by a sequence that is rich in acidic amino acids (the 'acid box') and appears essential for receptor function [17]. The interaction of ligand with the extracellular region of the receptor is, however, mediated by sequences present in both IgII and IgIII domains [18–20]. An important feature of the IgIII domain in the FGFR1–FGFR3 proteins is that it is encoded by three exons and produced by alternative splicing of a common 5′ exon, IgIIIa, onto either an IgIIIb or an IgIIIc variant exon. The significance of this alternative splicing in the IgIII domain is that it dictates ligand specificity. In the case of FGFR1 and FGFR2, the IgIIIa/IgIIIb form of the receptor binds to FGF-7 with high affinity, whereas the IgIIIa/IgIIIc

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form binds other members of the FGF family with high affinity [21,22]. In the case of FGFR3, the IgIIIa/IgIIIb variant preferentially binds to FGF-1, whereas the alternative variant IgIIIa/IgIIIc displays much broader binding specificity [23]. Although the description of *FGFR* expression (and splice variants) in development is by no means complete, there is enough evidence to suggest that each receptor isoform exhibits a characteristic pattern of expression in both embryonic and adult life, and many tissues and cells express a 'repertoire' of multiple FGF receptor genes and splice variants [24].

Animal models of FGF function

Clearly, FGF biology is potentially very complex, involving multiple ligands, receptors and cofactors, each expressed with different spatial and temporal patterns and distinct kinetics in the course of normal development. Considerable effort has been expended on the creation of different types of animal model for the analysis of FGF function *in vivo*. These studies clearly indicate that FGF signalling is involved in a number of different processes at different stages of development.

Homozygous null mutations

Gene ablation by homologous recombination is regarded as a highly informative test of gene function. The consequences of genetic inactivation of both FGF ligands and FGF receptors are now beginning to be revealed (see Table 1). Although the effects of inactivation need to be examined for the complete set of ligands, receptors and cofactors, both alone and in combination, some preliminary conclusions can be drawn.

Firstly, it is instructive that inactivation of individual FGF ligands leads to quite distinct effects, ranging from peri-implantation lethality [25] to minor disturbances in hair development [26]. These data show that different FGF family members have discrete biological functions. The second conclusion is that FGF signalling is critical at very early developmental stages: homozygous *FGF-4* and *FGFR1* null mutations both cause early lethality. The fact that *FGF-4*-null mice [25] die earlier than *FGFR1*-null mice [27,28] indicates that one (or more) other member(s) of the FGF receptor family is also critical for early embryonic development. Although this finding clearly establishes the importance of FGF signalling in early development, it also precludes the use of gene ablation for the analysis of FGF-dependent pathways in later development and adulthood.

Table 1. Phenotype of homozygous null mutations in mice.

Gene	Phenotype	Reference
FGF-3	Recessive phenotype. Viable neonates. Kinked tail. Fused and abnormally shaped caudal vertebrae. Defective development of inner ear.	[52]
FGF-4	Recessive phenotype. Embryonic lethal. Failure to develop post-implantation.	[25]
FGF-5	Recessive phenotype. Viable neonates. Abnormally long hair. Allelic with *go* (*angora*) mutation.	[26]
FGFR1	Recessive phenotype. Embryonic lethal. Gastrulation defects. Disruption of mesodermal patterning. Growth retardation.	[27]
FGFR1	Recessive phenotype. Embryonic lethal. Growth retardation. Defective axial mesoderm.	[28]

Dominant-negative receptors

An alternative approach to the analysis of FGF receptor action, which has some advantages over gene ablation for the dissection of function, is the use of dominant-negative *FGFR* mutants. These encode mutant receptors with truncated cytoplasmic domains, which inhibit the signalling mediated by resident wild-type receptors by forming inactive heterodimers in the presence of ligand [29]. Dominant-negative FGF receptors have been used with some success in the analysis of amphibian early development [30]. A potentially useful feature of dominant-negative receptors is that, because their activity depends upon interaction with the ligand, their action specifically reveals aspects of receptor function that require both activation of intracellular signalling and interaction with the ligand.

Two studies have been reported in which dominant-negative FGF receptors expressed under the control of strong tissue-specific promoters in transgenic mice have been used to investigate FGF function in later development. In one case, the human surfactant protein C promoter was used to target expression of a dominant-negative *FGFR2* (IgIIIb variant) exclusively to lung-bud epithelium in transgenic mice [31]. This resulted in lethality: newborn mice expressing the transgene had, in place of lungs, two undifferentiated and unbranched epithelial tubes that extended from the bifurcation of the trachea down to the diaphragm. In the second case [32], a dominant-negative *FGFR2* (IgIIIb variant) construct was targeted to suprabasal keratinocytes using a keratin 10 promoter. Expression of the mutant receptor disrupted the organization of epidermal keratinocytes, induced epidermal thickening and resulted in aberrant expression of keratin 6. These findings reveal that FGF signalling is required for both branching morphogenesis of the lung and the establishment of the normal program of keratinocyte differentiation in the skin. It seems very likely that these are just two examples of a diversity of FGF signalling functions in morphogenesis and differentiation.

Targeted overexpression

A complementary approach to analyzing FGF function is to examine the consequences of activating FGF signalling. This is usually achieved by ectopic application or expression of FGF ligands and reveals FGF-dependent signalling pathways that are controlled by the availability

of ligand. The strategy has been used to most effect in the analysis of limb development. In particular, FGF signalling has been shown to be involved in both the initial induction and sustained outgrowth of the limb bud during early limb development [33]. Perhaps the most dramatic illustration of this function of FGF signalling is the ability to induce supernumerary limb development in the chick by local application of an FGF-soaked bead [34]. These findings show that FGF has a major role in both specification and development of the limb, and that at least some FGF-dependent processes are regulated by accessibility of an FGF ligand.

FGF receptor mutations in human genetic disease

In the light of the evidence from animal models, it is clear that either ablation or activation of FGF signalling can have major harmful effects in embryonic development. It would therefore seem unlikely that alteration of FGF receptor function could lead to a viable adult phenotype. It is of considerable interest therefore that mutations in FGF receptor genes have, in the last year, been identified as the underlying causes of several human disorders of bone growth and patterning. The initial clue to these discoveries came from genetic linkage analysis in three human disorders: Pfeiffer syndrome, Crouzon syndrome and achondroplasia. These were localized to chromosomes 8p, 10q and 4p, respectively. The *FGFR* genes 1, 2 and 3 had previously been mapped to these regions by physical methods, and subsequent searches for mutations in these genes [35–38] in affected individuals have dramatically borne fruit.

The first gene to yield mutations was *FGFR3*. An identical heterozygous Gly→Arg substitution in the transmembrane domain of FGFR3 was identified in all 39 patients examined with achondroplasia [37,38]. A heterozygous Gly→Cys substitution located five residues away in the same domain has also been reported recently [39]. Achondroplasia is the most common genetic form of dwarfism, and is characterized by disproportionate shortening of the long bones of the limbs due to failure of cell proliferation at the epiphyseal plates during skeletogenesis. Homozygotes are very severely affected and usually die before, or shortly after, birth. A potential explanation of the effects of this mutation is the ligand-independent activation of receptor signalling as a result of dimerization of the mutant receptors, mediated by the mutant transmembrane domain. An analogous mechanism of receptor activation has been advanced in the case of the Her-2 receptor [40].

The hallmark of both Pfeiffer and Crouzon syndromes is craniosynostosis, an abnormality of the developing skull in which the sutures between the growing bones fail to form or fuse prematurely (Fig. 1a). This leads to distortions in the shape of the skull, sometimes associated with bulging eyes, enlarged cerebral ventricles and breathing problems. Although the facial features of Pfeiffer and Crouzon syndromes are quite similar, they can be differentiated by the pattern of limb involvement. Crouzon patients have normal hands and feet, whereas in Pfeiffer syndrome the thumbs and big toes are broad, and mild webbing between the digits may occur. Analysis of five families with Pfeiffer syndrome revealed a specific Pro→Arg substitution in the IgIIIa exon of *FGFR1* [35]. Further studies of *FGFR* mutations in Pfeiffer patients have uncovered a second class of mutation, in the IgIIIc exon of *FGFR2* [41,42]. In 13 published and 10 unpublished cases of Crouzon syndrome, a variety of mutations has been revealed in the IgIIIa and IgIIIc exons of *FGFR2*, including some at identical residues to those identified in Pfeiffer syndrome (Table 2).

More recently, two further craniosynostosis syndromes have been associated with *FGFR2* mutations. Apert syndrome is characterized not only by craniosynostosis but also by severe bony syndactyly of the hands and feet (Fig. 1b). A series of 40 Apert patients were identified as harbouring mutations in the IgIIIa exon of *FGFR2* [43]. Finally, Jackson–Weiss syndrome is a rare condition, described in a large Amish pedigree, which involves craniosynostosis in association with fusion between the bones of the feet. This family has a specific mutation in the IgIIIc exon of *FGFR2* [44].

Our current understanding of the spectrum of human mutations in FGF receptor genes is summarized in Figure 2. Viewed together, the four craniosynostosis syndromes that are associated with mutations in *FGFR1* and

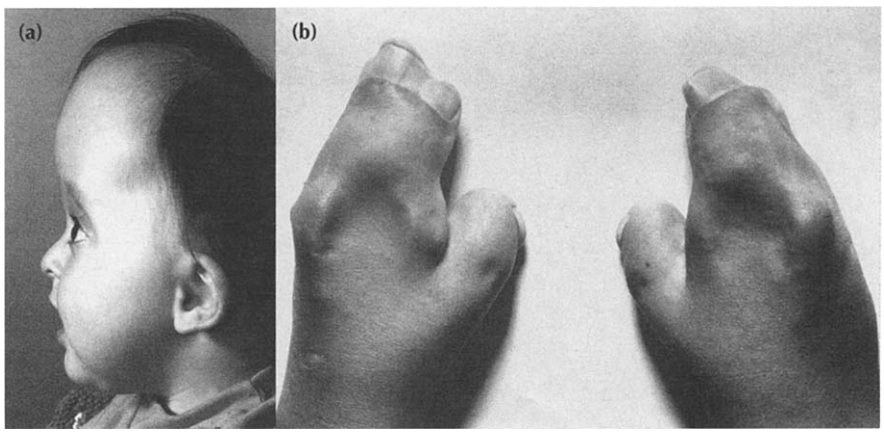

Fig. 1. **(a)** Crouzon syndrome. The tall forehead and anterio-posterior flattening of the skull profile are due to premature fusion of the coronal (transverse) cranial suture. **(b)** Apert syndrome. The three central digits of the hands show severe syndactyly, with a single transverse nail and underlying bony fusion of the phalanges.

Table 2. FGF receptor gene mutations in human disorders.

Syndrome	Gene/location	Mutation	Reference
Crouzon	*FGFR2*/IgIIIa	$Ser^{267}\rightarrow Pro$	*
		$Cys^{278}\rightarrow Phe$	*
		$Gln^{289}\rightarrow Pro$	*
	FGFR2/IgIIIc	$Tyr^{328}\rightarrow Cys$	[44]
		$Tyr^{340}\rightarrow His$	[36,44]
		$Cys^{342}\rightarrow Tyr$	[36]
		$Cys^{342}\rightarrow Arg$	[36]
		$Cys^{342}\rightarrow Ser$	[36]
		$Cys^{342}\rightarrow Phe$	[*]
		$Ala^{344}\rightarrow Ala^{\dagger}$	[36,44]
		$Ser^{347}\rightarrow Cys$	[44]
		$Ser^{354}\rightarrow Cys$	[36]
Pfeiffer	*FGFR1*/IgIIIa	$Pro^{252}\rightarrow Arg$	[35]
	FGFR2/IgIIIc	$Asp^{321}\rightarrow Ala$	[42]
		$Thr^{341}\rightarrow Pro$	[41]
		$Cys^{342}\rightarrow Arg$	[41]
		$Cys^{342}\rightarrow Tyr$	[41]
	FGFR2/intron preceding exon IIIc	A→G	[42]
Apert	*FGFR2*/IgIIIa	$Ser^{252}\rightarrow Trp$	[43]
		$Pro^{253}\rightarrow Arg$	[43]
Jackson–Weiss	*FGFR2*/IgIIIc	$Ala^{344}\rightarrow Gly$	[44]
Achondroplasia	*FGFR3*/ transmembrane	$Gly^{375}\rightarrow Cys$	[39]
		$Gly^{380}\rightarrow Arg$	[37,38]

* A.O.M.W., unpublished observations. † This synonymous substitution may alter splicing.

FGFR2 exhibit some important similarities. Firstly, all are manifest in individuals heterozygous for the mutation; the mutant alleles therefore exhibit dominance. The phenotype of homozygous mutant individuals is unknown, but homozygosity may well be lethal. Secondly, all four syndromes involve the common feature of craniosynostosis, but with variable involvement and severity of syndactyly in the hands and feet. Thirdly, all the mutations map to two specific regions of *FGFR1* and *FGFR2*: the IgIIIa and IIIc exons and a specific Ser–Pro motif found at the 5′ end of the IgIIIa exon. The discovery of this set of human *FGFR* mutations, each of which has significant, but non-lethal, biological consequences, has important implications for understanding FGF receptor design and function.

Implications for FGF receptor function

On the basis of information gleaned from the animal models of FGF dysfunction described above, it seems unlikely that the human mutations simply involve loss of receptor function; there have been no reports, for example, of craniosynostosis in $FGFR1^{-/+}$ heterozygous animals. It seems equally unlikely that the mutations involve gain-of function dominant-negative receptor mutations; these are usually associated with mutations in the cytoplasmic domain of a receptor which affect signal transduction.

A complete understanding of the function of craniosynostosis-associated mutations requires knowledge of the three dimensional structure of FGF receptors, and this information is currently lacking. A provisional model of FGF receptor structure can, however, be built on the basis of known structures of members of the immunoglobulin

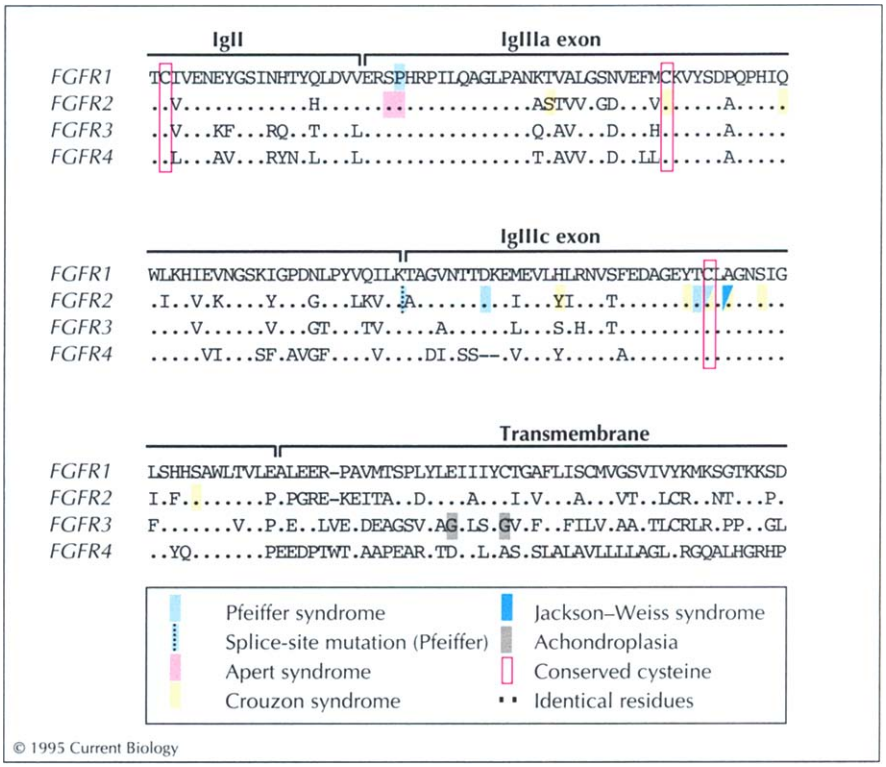

Fig. 2. Mutations of three FGF receptor genes in five human disorders. The portion of each *FGFR* that is shown comprises IgIIIa and IgIIIc, flanked by parts of IgII and the transmembrane domain.

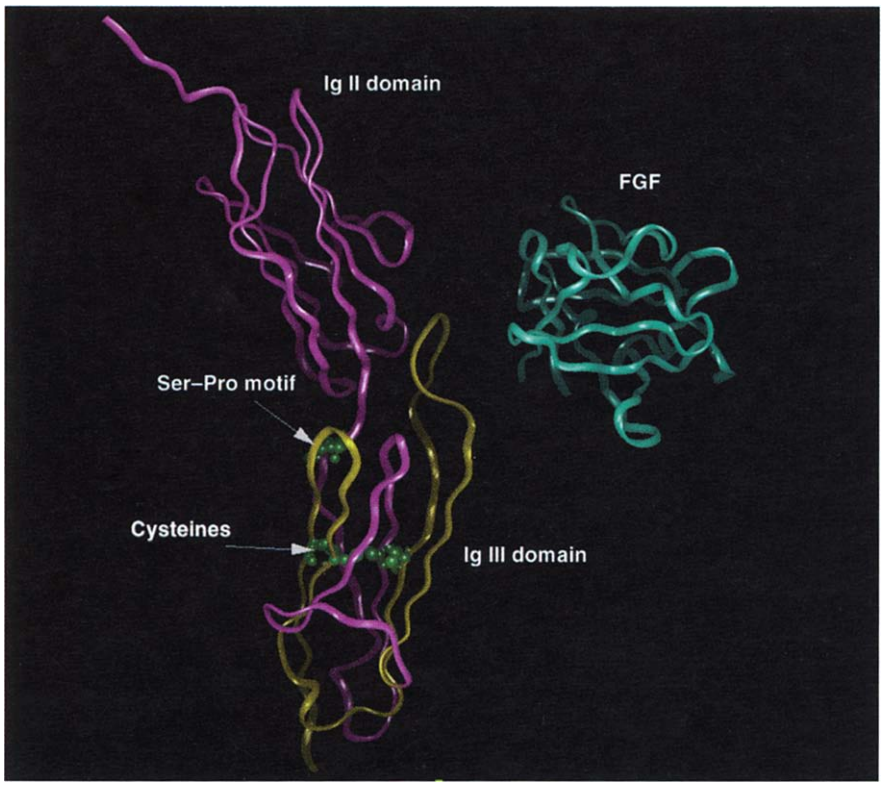

Fig. 3. Crystal structure of FGF and homology model of FGF receptor domains IgII and IgIII. The crystal structure of FGF-2 is that of Zhu *et al.* [2]. By sequence analysis, the IgII and IgIII domains of the FGF receptor are predicted to conform to the topology of I-set immunoglobulin domains ([53] and C. Chothia, personal communication), represented here by the crystal structure of telokin. The inter-domain linker sequence shows sequence similarity to that of the VCAM domain 1–2 linker. The linker and relative domain orientation is therefore modelled on the crystal structure of VCAMd1,2 [54]. Residues predicted to be encoded by the variant exons IIIb and IIIc are coloured in yellow and include the DE loop, which represents a major insertion in the FGF receptor compared to known structures: the current model merely indicates the relative size of such a loop. The remainder of the molecule is shown in pink, and the serine, proline and cysteine residues discussed in the text are shown in green Modelling was carried out using the graphics package FRODO [55]. The orientation of FGF-2 is based upon the identification of residues involved in receptor binding [56].

superfamily. Such a model for the pair of immunoglobulin domains implicated in ligand binding, IgII and IgIII, is presented in Figure 3. On the basis of this model, the two principal target regions for mutation in craniosynostosis-associated syndromes lie in quite distinct locations in the three-dimensional structure and have, despite the overall similarity in phenotype, somewhat different implications for the mechanism of mutant receptor action.

A notable feature of many IgIII mutations is the loss or creation of cysteine residues. A buried pair of disulphide-bonded cysteines is a frequent (but not ubiquitous) feature of immunoglobulin superfamily members [16]. Two of the target residues, Cys 278 and Cys 342, are predicted to be involved in this canonical bond. These two residues are conserved throughout the FGF receptor family, suggesting that they are important for structural stability. Indeed, mutations in the equivalent residues of FGFR1 have been shown to abolish ligand binding [20]. Mutation of one partner in the bond would have two consequences: first, the possibility of local unfolding of the IgIII domain (especially in the case of mutation to a polar amino acid), and second, the creation of a free, unpaired cysteine residue. The significance of unbonded cysteine is also suggested by the existence of other IgIII mutants in which residues, predicted by model building to be exposed, are mutated to cysteine. It is notable that mutations at other sites in the IgIII domain (such as Tyr 340) either involve residues that are both highly conserved amongst all FGFRs and predicted to play a key structural role in the immunoglobulin fold, or introduce proline residues that may inhibit correct folding. It is conceivable that these mutations not only disrupt the tertiary structure of IgIII but also destabilize the disulphide bond that is predicted to exist between Cys 278 and Cys 342.

The creation of free, exposed cysteine residues as a direct, or indirect, consequence of mutation suggests a possible mechanism for the dominant phenotypic effects of IgIII mutations: FGF receptor dimerization by intermolecular disulphide bonding leading to ligand-independent activation. There are precedents for this mechanism of activation in both the erythropoietin and the epidermal growth factor (EGF) receptors, in each of which ligand-independent activating mutations have been created by introduction of a free cysteine [45,46]. In addition, *MEN-2A* mutations of the *RET* gene involve point mutation of cysteine residues in the extracellular domain of the encoded protein and result in oncogenic activation [47]. Ligand-mediated receptor activation by intermolecular disulphide bonding has also been invoked in the action of interleukin 6 [48]. This analysis would lead to the proposal that craniosynostosis-associated IgIII mutations lead to ligand-independent receptor signalling. It is also conceivable that intermolecular disulphide bonding between mutant receptors and the free exposed cysteine residues in the FGF ligand leads to inappropriate receptor oligomerization.

The second target site for *FGFR* mutation is a pair of residues encoded by the 5′ end of the IgIIIa exon: Ser 252 and Pro 253 (*FGFR2* numbering). This pair of residues is highly conserved in the *FGFR* family and has been found to be mutated in both *FGFR2* and (at the corresponding proline residue) *FGFR1*. Modelling studies (Fig. 3) suggest that these residues are located in a short linker that

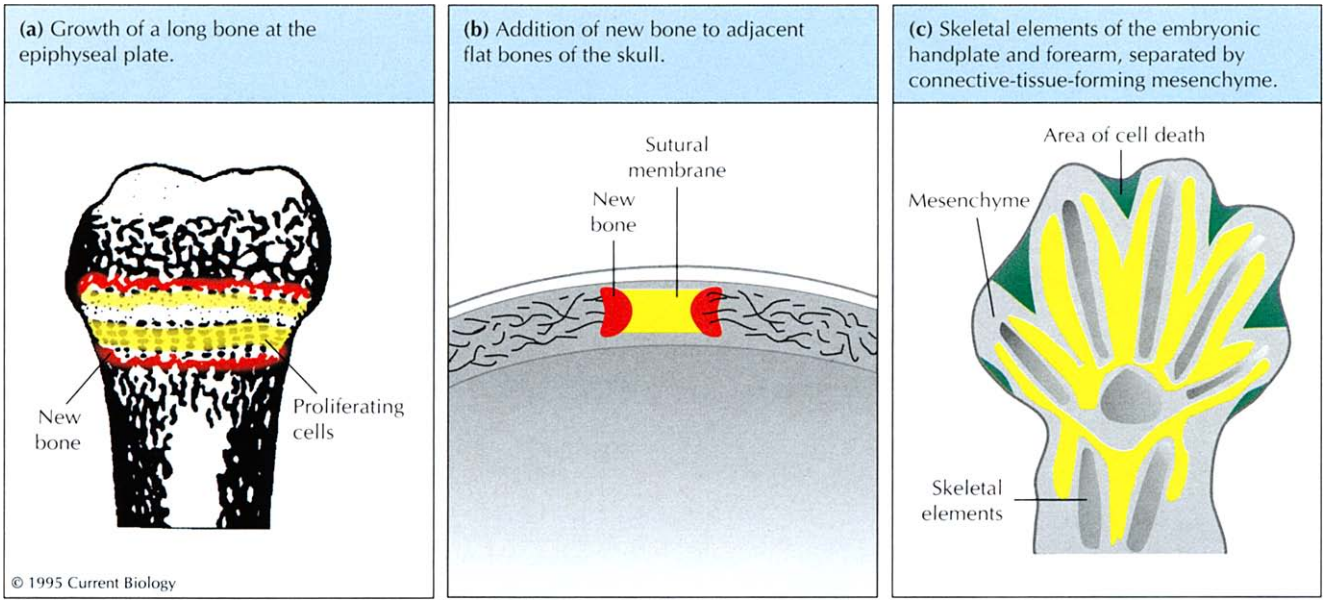

Fig. 4. Normal tissue organization sites that are affected by FGF receptor mutations.

connects the IgII and IgIII domains. It is not immediately obvious why mutations in this region should have biological consequences, but the fact that mutations in this region and in exon IgIIIc (discussed above) both result in craniosynostosis suggests that a similar functional disturbance is induced by two different strategies. This could, in particular, include changes to the inter-domain conformation which mimic or accentuate the effects of ligand binding. The significance of inter-domain orientation for ligand engagement has recently been highlighted in studies that compare the interaction of growth hormone with either the growth hormone receptor [49] or the prolactin receptor [50]. The identification of mutations in the inter-domain region of the FGF receptor may have important consequences for understanding the mechanism of interaction between the FGF ligand and wild-type receptor.

Structural and genetic considerations, therefore, point to the possibility that craniosynostosis mutations involve activation of FGF receptor signalling. This possibility is consistent with an additional piece of evidence. The *Bulgy eye* mutation in the mouse results from a retroviral insertion event between the genes encoding FGF-3 and FGF-4 (M. Carlton and M. Evans, personal communication). The phenotype of *Bulgy eye* mice is strongly reminiscent of human craniosynostosis syndromes, and it is tempting to suggest that it arises as a result of ectopic activation of FGF receptor signalling mediated by FGF-3 or FGF-4.

Biological specificity

A provisional explanation for the phenotype of human *FGFR* mutations is, therefore, that they represent a variety of different schemes for activating receptor signalling. This explanation would lead to the prediction that the biological consequences result from an excess of FGF-mediated functions. The currently available embryological data suggest that major and deleterious consequences would arise in embryonic life from widespread activation of FGF receptor signalling. The many precedents of activating mutations in intrinsic tyrosine-kinase receptors would also suggest a predisposition to tumourigenesis in patients with *FGFR* mutations, which has not been detected. This analysis, therefore, fails to explain the biological basis or specificity of the craniosynostosis or achondroplastic *FGFR* mutations.

The growth of skeletal elements, and their maintenance as discrete individual structures separate from adjacent elements, depends upon a balance between cell proliferation and differentiation. Cell proliferation occurs at specific sites within the developing skeleton, such as the epiphyseal plates of long bones and the sutures of the skull. These sites are eventually lost to ossification at precise stages of postnatal life as the epiphyses and sutures fuse (Fig. 4). The separation of adjacent skeletal elements in the hand or foot may be analogous, being characterized by the presence of undifferentiated proliferating mesenchyme between the nascent metacarpals or metatarsals and the proximal parts of the digits. In the hand and foot, however, the developmental fate of interskeletal mesenchyme is partly apoptosis but mainly the formation of connective tissue, which maintains a permanent separation of the skeletal elements. In the case of Apert syndrome, the failure of skeletal elements to be maintained as separate structures continues into adult life, with progressive skeletal fusions. Analysis of existing syndactylous mouse mutants, such as *Oligosyndactylism* [51], suggests that deficiency of interdigital mesenchyme from an early stage leads to premature fusion of adjacent areas of condensing precartilaginous mesenchyme.

Achondroplasia, craniosynostosis and syndactyly therefore appear to result from failure or insufficiency of specific

areas of interskeletal tissue in the execution of proliferative (or anti-differentiative) functions. The molecular functions of mutant FGF receptors must accordingly be understood in this light. The specificity of the phenotypes arising from *FGFR* mutation also requires that the regulation of interskeletal tissue function by FGFs exhibits some special, and hitherto unexpected, biological properties. The new findings from clinical molecular genetics have therefore raised important issues for both FGF receptor function and skeletal biology. The challenge now is to complete the connections between knowledge of altered genotype and the molecular basis of altered phenotype.

Acknowledgements: A.O.M.W. and J.K.H. are supported by the Wellcome Trust. E.Y.J. is a Royal Society University Research Fellow. We are grateful to S. Slaney for the photograph of Apert syndrome, M. Oldridge for assistance and C. Chothia for helpful suggestions on FGF receptor structure.

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Received: **2 March 1995.**